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APPLICATION FOR LETTERS PATENT

for

CORONA-VIRUS-LIKE PARTICLES COMPRISING FUNCTIONALLY DELETED GENOMES

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TITLE OF THE INVENTION

CORONA-VIRUS-LIKE PARTICLES COMPRISING FUNCTIONALLY DELETED GENOMES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of co-pending application, serial no. 10/714,534, filed November 14, 2003, and a continuation-in-part of co-pending application serial no. 10/414,256, filed April 14, 2003, which are both continuations of PCT International Patent Application No. PCT/NL/02/00318, filed May 17 2002, designating the United States of America, and published, in English, as PCT International Publication No. WO 02/092827 A2 on November 21, 2002, the contents of the entirety of all of which are incorporated by this reference.

TECHNICAL FIELD

The invention relates generally to biotechnology, and more specifically to the field of coronaviruses and diagnosis, therapeutic use and associated vaccines.

BACKGROUND

Coronavirions have a rather simple structure. They consist of a nucleocapsid surrounded by a lipid membrane. The helical nucleocapsid is composed of the RNA genome packaged by one type of protein, the nucleocapsid protein N. The viral envelope generally contains 3 membrane proteins: the spike protein (S), the membrane protein (M) and the envelope protein (E). Some coronaviruses have a fourth protein in their membrane, the hemaglutinin-esterase protein (HE). Like all viruses coronaviruses encode a wide variety of different gene products and proteins. Most important among these are obviously the proteins responsible for functions related to viral replication and virion structure. But besides these elementary functions viruses generally specify a diverse collection of proteins the function of which is often still unknown but which are known or assumed to be in some way beneficial to the virus. These proteins may either be essential - operationally defined as being required for virus replication in cell culture - or dispensable. Coronaviruses constitute a family of large,

positive-sense RNA viruses that usually cause respiratory and intestinal infections in many different species. Based on antigenic, genetic and structural protein criteria they have been divided into three distinct groups: group I, II and III. Actually, in view of the great differences between the groups their classification into three different genera is presently being discussed by the responsible ICTV Study Group. The features that all these viruses have in common are a characteristic set of essential genes encoding replication and structural functions. Interspersed between and flanking these genes sequences occur that differ profoundly among the groups and that are, more or less, specific for each group.

To successfully initiate an infection, viruses need to overcome the cell membrane barrier. Enveloped viruses achieve this by membrane fusion, a process mediated by specialized viral fusion proteins. Most viral fusion proteins are expressed as precursor proteins, which are endoproteolytically cleaved by cellular proteases giving rise to a metastable complex of a receptor binding and a membrane fusion subunit. The present invention provides methods and means to interfere with fusion of corona viruses. According to the invention after receptor binding at the cell membrane, the fusion proteins undergo a dramatic conformational transition. A hydrophobic fusion peptide becomes exposed and inserts into the target membrane. The free energy released upon subsequent refolding of the fusion protein to its most stable conformation is believed not only to facilitate the close apposition of viral and cellular membranes but also to effect the actual membrane merger (1, 46, 54).

SUMMARY OF THE INVENTION

The present invention provides methods and means to use the biochemical and functional characteristics of the heptad repeat (HR) regions of the corona virus spike proteins. We show here that peptides corresponding to the HR regions assemble into a thermostable, oligomeric, alpha-helical rod-like complex, with the HR1 and HR2 helices oriented in an anti-parallel manner.

Furthermore, we have found that HR2 of the corona virus spike protein such as MHV-A59 spike protein is a strong inhibitor of both virus-cell and cell-cell fusion.

The invention also provides the amino acid sequences of the HR regions of a coronavirus belonging to another group such as Feline infectious peritonitis (FIP) virus spike protein, and of the inhibition of cell-to-cell fusion in FIPV infected cells by administration of HR2 of viruses such as FIPV. We demonstrate that the same mechanism is valid in different groups of coronaviruses.

The present invention also provides the amino acid sequences of the HR regions of the spike protein of a coronavirus, which causes a severe acute respiratory syndrome (SARS) in humans and which has been designated provisionally as SARS coronavirus (SARS-CoV). The inhibitory effect of SARS-CoV HR derived peptides on infection of cells by SARS-CoV is also disclosed, and peptides have been identified that can be used as a vaccine against SARS-CoV infections or for the preparation of a medicine against a SARS-CoV caused disease. In addition, this invention discloses the amino acid sequence of the fusion peptide of SARS-CoV. The fusion peptide can also be used as a vaccine against SARS-CoV infections or for the preparation of a medicament against a SARS-CoV caused disease.

The invention makes use of the discovery that in coronaviruses the energy necessary for the membrane fusion process is at least partly provided by the formation of an anti-parallel coiled coil structure by folding of the spike protein and interaction of the HR1 and HR2 repeat region. Decreasing the contact of the heptad repeat regions in the spike protein results in a less optimal fit of the coiled coil and thus in less energy for the fusion of membranes. Therefore, this invention teaches a method for at least in part inhibiting anti-parallel coiled coil formation of a coronavirus spike protein comprising decreasing the contact between heptad repeat regions of the protein. Of course, blocking the coiled coil formation by occupying the sequence of either HR1 or HR2 is a good way of decreasing, or even preventing coiled coil formation,

The contact of the heptad repeat regions can be disturbed by a molecule or compound that binds to HR1 or HR2 and by binding to these regions, or in close proximity, the compound blocks the site for binding to another HR site. This will result in decreasing or inhibiting the ability of the coronavirus to fuse with a membrane and enter a cell. Of course, if binding of a compound occurs in the vicinity of these regions, contact of the heptad repeat regions may also be decreased and/or inhibited. Such a compound may for example be a

peptide and/or a functional fragment and/or an equivalent thereof with an amino acid sequence as shown in FIG. 1.

A functional fragment of a protein or peptide is defined as a part which has the same kind of biological properties in kind, not necessarily in amount. A "functional equivalent" of a peptide is defined as a compound be it a peptide or proteinaceous or non-proteinaceous molecule with essentially the same functional properties in kind, not necessarily in amount. A functional equivalent can be provided in many ways, for instance through conservative amino acid substitution.

A person skilled in the art is well able to generate analogous equivalents of a protein. This can for instance be done through screening of a peptide library. Such an equivalent has essentially the same biological properties of the protein or peptide in kind, not necessarily in amount.

Therefore, this invention teaches a method for at least in part inhibiting anti-parallel coiled coil formation of a coronavirus spike protein comprising decreasing the contact between heptad repeat regions of the protein, wherein the decreasing is provided by a peptide and/or a functional fragment and/or an equivalent thereof.

Decreasing the contact between heptad regions may also be provided by a peptide comprising a heptad repeat region of a coronaviral spike protein and/or a functional fragment and/or an equivalent thereof. Therefore, this invention provides a method to decrease and/or inhibit contact between heptad regions wherein the decreasing and/or inhibiting is provided by a peptide comprising a heptad repeat region of a coronaviral spike protein and/or a functional fragment and/or an equivalent thereof. The disclosure of the amino acid sequence of HR2 of SARS-CoV enables the production and/or selection of peptides comprising SARS-CoV HR2 of spike protein and/or a functional fragment and/or an equivalent thereof.

In another embodiment, such decreasing can be achieved by providing an antibody directed against a part of HR1 or HR2. The antibody will inhibit the binding of a heptad repeat region to another heptad repeat region, thus preventing at least in part the formation of an anti-parallel coiled coil. Of course, binding of an antibody to a region in close proximity to the heptad region may also disturb the correct fit of the heptad repeat regions in a coiled coil. Therefore, the present invention teaches a method for at least in part inhibiting anti-parallel

coiled coil formation of a coronavirus spike protein comprising decreasing the contact between heptad repeat regions of the protein, wherein the decreasing is provided by an antibody and/or a functional fragment and/or an equivalent thereof.

The present invention shows comparative data on the amino acid sequences of the HR1and HR2 region of a number of coronaviruses and of SARS coronavirus (FIG. 1). The human coronavirus HCV-229E and the feline infectious peritonitis virus (FIPV), which both belong to the group 1 coronaviruses show an insertion of 14 amino acids in the HR1 and in the HR2 region, which the other coronaviruses like mouse hepatitis virus and another human coronavirus (HCV-OC43) (group 2), and infectious bronchitis virus of poultry (group 3) and SARS-CoV do not have. This insertion of 14 amino acids in each heptad region may generate more electrostatic power for the fusion of a membrane, once the coiled-coil is formed, because the total length of each heptad alpha helix is elongated by 2 coils. The fact that FIPV and HCV-229E have these extra 2 coils per heptad repeat region may indicate that these viruses need extra energy to fuse their membrane with that of their host cell. Decreasing this energy by inhibiting at least in part the formation of a coiled coil will effectively decrease the penetrating power of the viruses. Therefore, the invention teaches a method for at least in part inhibiting anti-parallel coiled coil formation of a coronavirus spike protein comprising decreasing the contact between heptad repeat regions of the protein, wherein the coronavirus comprises a feline coronavirus and/or a human coronavirus, and/or a mouse hepatitis virus MHV and/or a SARS virus.

After infection of a cell by a coronavirus, the infected cell exhibits coronaviral spike protein on its surface. Coronaviral spike protein present on the cell membrane surface mediates the fusion of cell membranes of other cells, thus allowing cell-to-cell fusion and allowing the virus to passage from the infected cell to a neighboring cell without the need to leave the cell. An important step in decreasing viral infection of cells is by preventing the cell-to-cell fusion. By providing a compound such as a peptide or an antibody that decreases and/or inhibits the contact of heptad regions, cell-to-cell fusion will be decreased and/or inhibited. The present invention teaches a method for inhibiting coronavirus spike protein mediated cell-to-cell fusion, comprising decreasing and/or inhibiting the contact between heptad repeat regions of the spike protein.

The present invention also provides methods for selecting further inhibitors of coiled coil formation in corona viruses. For example, the HR1 and HR2 peptides may be used *in vitro* to select binding compounds from libraries of molecules. Any compound that binds to at least part of an HR1 or HR2 peptide is selected and is used as an inhibitor of the formation of an anti-parallel coiled coil in a spike protein of coronavirus. Therefore, this invention teaches a method to select a compound binding to a heptad repeat region of a coronavirus spike protein, comprising contacting *in vitro* at least one heptad region of a coronavirus spike protein with a collection of compounds and measuring the formation of an anti-parallel coiled coil in the protein.

The present invention also teaches a compound selected by contacting *in vitro* at least one heptad region of a coronavirus spike protein with a collection of compounds and measuring the formation of an anti-parallel coiled coil in the protein. With this method, non-proteinaceous compounds, proteinaceous compounds and antibodies are selected for their capacity to bind to the heptad repeat regions. Of course, a functional fragment and/or derivative of an antibody may also bind to heptad repeat regions. Therefore, this invention also teaches an antibody or a functional fragment and/or derivative thereof, capable of decreasing and/or inhibiting the contact between heptad repeat regions of a coronavirus spike protein. The abovementioned compounds and/or antibodies may be incorporated into a pharmaceutical composition with a suitable diluent and/or or carrier compound. Therefore, the invention teaches a pharmaceutical composition comprising the compound and/or the antibody or a functional fragment and/or derivative thereof, and a suitable diluent and/ or carrier. Administration of the pharmaceutical composition to a cell or a subject with a coronaviral infection will inhibit the infection of cells and at least in part decrease the coronaviral infection. Therefore, the invention teaches a method of treatment of coronavirus infections comprising providing to a subject the pharmaceutical composition.

In another embodiment, the compounds and/or antibodies may be used to detect the presence of coronavirus in a cell or in a subject by contacting a sample of the cells or of the subject to the compound or the antibody and visualizing any binding of the coronavirus to the compound and/or the antibody. The visualizing may be performed by any method known in the art, for example by ELISA techniques or by fluorescence or histochemistry. Therefore,

the present invention also teaches a diagnostic kit for detecting coronavirus infection in a sample of a subject comprising the compound or the antibody, further comprising a means of detecting binding of the compound or antibody to the coronavirus. In yet another embodiment, the compound may be used to measure antibody titers of a subject. This may be done to diagnose whether a subject is undergoing a coronaviral infection, or has undergone a coronaviral infection in the past. This may be useful, not only for diagnostic purposes, but also for assessing the possible risk of a subject for a coronaviral infection, and for evaluating vaccination efficiency and strategy. Therefore, the present invention also teaches a diagnostic kit for detecting coronavirus antibodies in a sample of a subject comprising the compound, further comprising a means of detecting binding of the compound to the antibodies.

In another embodiment, the amino acid sequence of the heptad repeat regions is manipulated by recombination, insertion, or deletion techniques that are known in the art. Such a manipulation of the coronaviral genome in or around the heptad repeat regions will result in decreased and/or inhibited contact of the heptad repeat regions; it will result in attenuation of the coronavirus. Therefore, the invention teaches a method to attenuate a coronavirus comprising decreasing and/or inhibited the contact between heptad repeat regions of the spike protein of the coronavirus. The method enables the production of an attenuated coronavirus with a decreased contact between the heptad repeat regions. Therefore, the invention teaches an attenuated coronavirus characterized in that the contact between heptad repeat regions of the spike protein of the coronavirus is decreased and/or inhibited.

The invention also discloses a number of peptides derived from SARS-C0V HR2 region that inhibited infection of cells by SARS-CoV, therefore, the present invention discloses a method for at least in part inhibiting anti-parallel coiled coil formation of a coronavirus spike protein comprising decreasing the contact between heptad repeat regions of the protein, wherein the peptide comprises an amino acid sequence according to peptide sHR2-1,and/or sHR2-2, and/or sHR2-8, and/or sHR2-9 as described in FIG. 11 B, and/or a functional fragment and/or an equivalent thereof.

In another embodiment, the invention discloses amino acid sequences of the fusion peptide of SARS-CoV. Therefore, the present invention discloses a method for at least in part inhibiting anti-parallel coiled coil formation of a coronavirus spike protein comprising

decreasing the contact between heptad repeat regions of the protein, for at least in part inhibiting a fusion of a coronavirus with a cell membrane comprising decreasing binding of a fusion peptide with the cell membrane. Furthermore, the present invention discloses above described method, wherein the fusion peptide comprises the amino acid sequence of SARS-CoV as described in FIG. 17.

Because the fusion peptide of SARS-CoV is disclosed, inhibition of fusion may be used to find and select molecules that specifically bind to the fusion protein. Therefore, the present invention discloses the above described method, wherein the decreased binding is provided by a specific binding molecule for the fusion peptide. The disclosed fusion peptide is used to select antibodies and/or a functional fragment and/or a derivative thereof that specifically bind the fusion peptide, according to well known techniques in the art, such as for example phage display. Therefore, the present invention also discloses a method for at least in part inhibiting anti-parallel coiled coil formation of a coronavirus spike protein comprising decreasing the contact between heptad repeat regions of the protein, for at least in part inhibiting a fusion of a coronavirus with a cell membrane comprising decreasing binding of a fusion peptide with the cell membrane, wherein the specific binding molecule is an antibody and/or a functional fragment and/or a derivative thereof.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. (A) Schematic representation of the coronavirus spike protein structure. The glycoprotein has an N-terminal signal sequence (SS) and a transmembrane domain (TM) close to the C-terminus. Group 2 and 3 coronavirus spike proteins are proteolytically cleaved (arrow) in an S1 and an S2 subunit, which are non-covalently linked. S2 contains two heptad repeat regions (shaded bars), HR1 and HR2, as indicated. (B) Sequence alignment of HR1 and HR2 domains of the newly identified SARS-CoV (strain TOR2) with those of the group 1 coronaviruses FIPV (feline infectious peritonitis virus strain 79-1146) and HCoV-229E (human coronavirus strain 229E), the group 2 coronaviruses MHV-A59 (mouse hepatitis virus strain A59) and HCoV-OC43 (human coronavirus strain OC43), and the group 3 coronavirus IBV (infectious bronchitis virus strain Beaudette) (GenBank accession nos. P59594, VGIH79, VGIHHC, P11224, CAA83661 and P11223, respectively). Dark shading marks sequence

identity while lighter shading represents sequence similarity. The alignment shows a remarkable insertion of exactly two heptad repeats (14 a.a.) in both HR1 (SEQ ID NO:__) and HR2 (SEQ ID NO:__) of HCoV-229E (SEQ ID NO:__), and FIPV (SEQ ID NO:__), a characteristic of all group 1 viruses. The predicted hydrophobic heptad repeat 'a' and 'd' residues are indicated above the sequence. Asterisks denote conserved residues, dots represent similar residues. The amino acid sequences of the HR1 derived peptides HR1 (SEQ ID NO:__), HR1a (SEQ ID NO:__), HR1b (SEQ ID NO:__), HR1c (SEQ ID NO:__), and a FLAG-tagged HR1 (Fl.HR1) and of the HR2 derived peptides HR2 (SEQ ID NO:__), HR2-1 (SEQ ID NO:__), and a FLAG-tagged HR2 (Fl-HR2) (SEQ ID NO:__) of SARS-CoV (SEQ ID NO:__) used in this study are presented in italics below the alignments. N-terminal glycine and serine residues derived from the thrombin proteolytic cleavage site of the GST fusion protein are in parentheses.

FIG. 2. Hetero-oligomeric complex formation of HR1 and HR1a with HR2. (A) HR1 and HR2 on their own or as a preincubated equimolar (80 μM) mix were subjected to 15% tricine SDS-PAGE. Before gel loading, samples were either heated at 100°C or left at RT. Positions of HR1, HR2 and HR1-HR2 complex are indicated on the left, while the positions of molecular mass markers are indicated at the right. (B) Same as (A) but with peptide HR1a instead of HR1.

FIG. 3. Temperature stability of HR1-HR2 complex. An equimolar mix of HR1 and HR2 (80 μM) was incubated at RT for 1 h. Samples were subsequently heated for 5 min at the indicated temperatures in 1x tricine sample buffer and analyzed by SDS-PAGE in a 15% tricine gel, together with HR1 and HR2 alone. Positions of HR1, HR2 and HR1-HR2 complex are indicated on the left, while the molecular mass markers are indicated at the right.

FIG. 4. Circular dichroism spectra (mean residue eliplicity) of the HR1 (25 μM; open square) peptide, the HR2 (25 μM; filled triangle) peptide, and of the HR1-HR2 complex (25 μM; filled square) in water at RT. Note that the HR1 and HR2 spectra virtually coincide.

FIG. 5. Electron micrographs of HR1-HR2 complex.

FIG. 6. Proteinase K treatment of HR peptides. The peptides HR2, HR1, HR1a, HR1b and HR1c were subjected to Proteinase K either individually in solution or after mixing of the different HR1 peptides with HR2 at equimolar concentration followed by 1 h incubation at

37°C. Proteolytic fragments were separated and purified by HPLC and characterized by mass spectrometry. Peptides are schematically indicated by bars. Hatched bars indicate the protease sensitive part(s) of the peptide. N and C-terminal position of the peptide and the amino acid numbering are indicated.

FIG. 7. Inhibition of virus-cell and cell-cell fusion by HR peptides. (A) Virus-cell inhibition by HR peptides using a luciferase gene expressing MHV. LR7 cells were inoculated with virus at an MOI of 5 in the presence of varying concentrations of peptide ranging from 0.4 – 50 μM. At 5 h p.i. cells were lysed and luciferase activity was measured. (B) Inhibition of spike mediated cell-cell fusion by HR peptides. BSR T7/5 effector cells - BHK cells constitutively expressing T7 RNA polymerase (3), were infected with vaccinia virus for 1 h and subsequently transfected with a plasmid containing the S gene under a T7 promoter. Three hours post transfection, LR7 target cells transfected with a plasmid carrying the luciferase gene behind a T7 promoter, were added to the effector cells. Cells were incubated for another 4 h in the presence or absence of HR peptide. Cells were lysed and luciferase activity was measured.

FIG. 8. Schematic representation (approximately to scale) of the viral fusion proteins of six different virus families; MHV-A59 S (*Coronaviridae*), Influenza HA (*Orthomyxoviridae*), HIV-1 gp160 (*Retroviridae*), SV5 F, (*Paramyxoviridae*), Ebola Gp2 (*Filoviridae*) and Se*M*NPV F (*Baculoviridae*). Cleavage sites are indicated by triangles; the black bars represent the (putative) fusion peptides, the vertically hatched bars the HR1 domains and the horizontally hatched bars the HR2 domains. Transmembrane domains are indicated by the vertical, dashed lines. For each polypeptide the total length is given at the right.

FIG. 9. GST-FIPV fusion protein sequences of GST-HR1 (SEQ ID NO:__) and GST-HR2 (SEQ ID NO:__).

FIG. 10 SARS nucleotide and deduced protein sequence as derived from the RT-PCR fragment (SEQ ID NO:__).

FIG. 11. Inhibition of SARS-CoV infection by HR peptides. (A) VERO cells were mock infected or infected with SARS-CoV (MOI = 0.5) in the presence of the HR2-1 peptide (sHR2-1) at concentrations of 0, 5, or 25 μM and incubated in medium containing the same

concentration of peptide. An infection in the presence of peptide (25 μM) corresponding to the HR2 domain of MHV (mHR2) was taken along as a negative control. At 16 h p.i., cells were fixed and SARS-CoV positive cells were visualized by immunofluorescence staining. The Table (panel B) shows amino acid sequences of HR2 (B1) (SEQ ID NOs:__-__ respectively) and HR1 (B2) (SEQ ID NOs:__-__ respectively) derived peptides of SARS-CoV (SCV) and MHV and their EC_{50} values as determined in a 96 wells format infection inhibition assay. (EC_{50}: 50% inhibitory concentration; SD: standard deviation).

FIG. 12. Complex formation of SARS-CoV HR1 and HR2 peptides. (A). Comparison of SARS-CoV and MHV. HR1 and HR2 peptides on their own or as a preincubated equimolar (100 μM) mixture were subjected to 15% Tricine SDS-PAGE. Just before loading onto the gel, some samples were heated at 100°C. (B) HR1-HR2 complex formation using FLAG-tagged and non-tagged SARS-CoV HR peptides. Samples of the individual peptides HR1 (1), HR2 (2), FLAG-tagged HR1 (F1) and FLAG-tagged HR2 (F2), and of preincubated mixtures of these peptides (1+2, F1+2, 1+F2 and F1+F2) were subjected to 15% Tricine SDS-PAGE. The positions of molecular mass markers are indicated at the left.

FIG. 13. Stoichiometry of peptides in HR1-HR2 complexes. (A) FLAG-tagged HR2 and non-tagged HR2 were mixed in different ratio's and incubated with an equimolar amount of HR1 to allow complex formation for 3 h followed by analysis in a 10% Tricine SDS-PAGE. (B) FLAG-tagged HR1, non-tagged HR1 and a 1:1 mixture of the two peptides were incubated with an equimolar amount of HR2 for 3 h and subsequently analyzed in a 10% Tricine SDS-PAGE. (C) Acetonitrile was added to a concentration of 50% (v/v) to solutions of FLAG-tagged HR1 (100 μM), non-tagged HR1 (100 μM) or to a 1:1 mixture of these two solutions. After mixing and incubation for 5 min, the acetonitrile was evaporated and an equimolar amount of HR2 was added to allow complex formation. After 3 h samples were analyzed in a 10% Tricine SDS-PAGE. Only the part of the gel containing the complexes is shown. The positions of molecular mass markers are indicated at the left.

FIG. 14. Comparative temperature stabilities of HR1-HR2 complexes of SARS-CoV and MHV. Equal amounts of SARS-CoV and MHV HR1-HR2 complexes were pooled, subsequently incubated for 5 min at the indicated temperatures in 1x Tricine sample buffer and analyzed directly by SDS-PAGE in a 15% Tricine gel. Positions of the HR1-HR2

complex of SARS-CoV and MHV are indicated on the right, while the molecular mass markers are indicated at the left.

FIG. 15. Circular dichroism spectra (mean residue eliplicity Φ) of the HR1 (20 μM; filled square) peptide, the HR2 (20 μM; open square) peptide, and of the HR1-HR2 complex (20 μM; filled triangle) in water at RT. Note that the three spectra virtually coincide.

FIG. 16. Proteolytic analysis of the HR1-HR2 complex. The peptides HR2 (SEQ ID NO:__), HR1a (SEQ ID NO:__) or preincubated equimolar mixtures of HR2 (SEQ ID NO:__) with HR1a (SEQ ID NO:__) or HR1c (SEQ ID NO:__) were subjected to Proteinase K (pK) digestion and analyzed by RP HPLC (upper part). The peaks representing the protected fragments were purified by RP HPLC. The molecular masses of the protected fragments were determined by mass spectrometry (lower part), allowing the identification of the protease-resistant cores of the peptides. The molecular masses of the protected fragments determined by mass spectrometry (Ms Mw) matched their predicted masses (Pred. Mw) within 1 Da.

FIG. 17. Hydrophobic domains in coronavirus spike proteins. The TMAP program was applied on a Clustal W alignment of nine coronavirus spike sequences (see Methods section). In the hydrophobicity plot obtained, the three predicted transmembrane domains are indicated by black bars (middle part). Arrows point to the corresponding hydrophobic regions in the schematic drawing of the spike protein (upper part), which represent the N-terminal signal sequence (SS), the C-terminal transmembrane anchor (TM) downstream of the HR2 domain, and the putative fusion peptide (FP) immediately upstream of the HR1 domain. In the bottom part of the figure the Clustal W multiple sequence alignment of this latter domain is shown for the nine coronavirus spike proteins (SEQ ID NOs:__-__, respectively).

DETAILED DESCRIPTION OF THE INVENTION

With a positive stranded RNA genome of 28-32 kb, the *Coronaviridae* are the largest enveloped RNA viruses. Coronaviruses exhibit a broad host range, infecting mammalian and avian species. They are responsible for a variety of acute and chronic diseases of the respiratory, hepatic, gastrointestinal and neurological systems (56).

Recently, coronavirus induced pneumonia (Severe Acute Respiratory Syndrome, SARS) has spread rapidly from China via Hong Kong to the rest of the world. The spike (S)

protein is the sole viral membrane protein responsible for cell entry. It binds to the receptor on the target cell and mediates subsequent virus-cell fusion (6). Spikes can be seen under the electron microscope as clear, 20 nm large, bulbous surface projections on the virion membrane (14). The spike protein of mouse hepatitis virus (MHV-A59) is a 180 kDa heavily N-glycosylated type I membrane protein which occurs in a homodimeric (37, 66) or homotrimeric (16) complex. In most murine hepatitis strains, the S protein is cleaved intracellularly into an N-terminal subunit (S1) and a membrane anchored subunit (S2) of similar size, which are non-covalently linked and have distinct functions. Binding to the MHV receptor (MHVR) (74) has been mapped to the N-terminal 330 amino acids (a.a.) of the S1 subunit (62), whereas the membrane fusion function resides in the S2 subunit (78). It has been suggested that the S1 subunit forms the globular head while the S2 subunit constitutes the stalk-like region of the spike (15). Binding of S1 to soluble MHVR, or exposure to 37°C and an elevated pH (pH 8.0) induces a conformational change which is accompanied by the separation of S1 and S2 and which might be involved in triggering membrane fusion (21, 27, 60). Cleavage of the S protein into S1 and S2 has been shown to enhance fusogenicity (25, 61) but cleavage is not absolutely required for fusion (2, 26, 59, 61).

The ectodomain of the S2 subunit contains two regions with a 4,3 hydrophobic (heptad) repeat (15), a sequence motif characteristic of coiled coils. These two heptad repeat (HR) regions, designated here as HR1 and HR2, are conserved in position and sequence among the members of the three coronavirus antigenic clusters (FIG. 1). A number of studies have shown that the HR1 and HR2 regions are involved in viral fusion. First, a putative internal fusion peptide has been proposed to occur close to (7) or within (40) the HR1 region. Second, viruses with mutations in the membrane-proximal HR2 region exhibited defects in spike oligomerization and in fusion ability (39). Third, it has been suggested that the MHV-4 (JHM) strain can utilize both endosomal and nonendosomal pathways for cell entry but does not require acidification of endosomes for fusion activation (48). However, mutations found in murine hepatitis viruses which do require a low pH for fusion, appeared to map to the HR1 region (23).

HR regions appear to be a common motif in many viral fusion proteins (57). There are usually two of them; one N-terminal HR region (HR1) adjacent to the fusion peptide and a C-

terminal HR region (HR2) close to the transmembrane anchor. Structural studies on viral fusion proteins reveal that the HR regions form a six-helix bundle structure implicated in viral entry (reviewed in (18). The structure consists of a homotrimeric coiled coil of HR1 domains in the exposed hydrophobic grooves of which the HR2 regions are packed in an anti-parallel manner. This conformation brings the N-terminal fusion peptide in close proximity to the transmembrane anchor. Because the fusion peptide inserts into the cell membrane during the fusion event, such a conformation facilitates a close apposition of the cellular and viral membrane (reviewed in (18)). Recent evidence suggests that the actual six-helix bundle formation is directly coupled to the merging of the membranes (46, 54). The similarities in the structures of the six-helix bundle complexes elucidated for influenza virus HA (4, 11), human and simian immunodeficiency virus (HIV-1, SIV) gp41 (5, 8, 41, 63, 69, 76), Moloney murine leukemia virus typel (MoMLV) gp21 (19), Ebola virus GP2 (42, 68), human T-cell leukemia virus type I (HTLV-1) gp21 (32), Visna virus TM, (43), simian parainfluenza virus (SV5) F1 (1), and human respiratory syncytial virus (HRSV) F1 (80), all point to a common fusion mechanism for these viruses.

Based on structural similarities, two classes of viral fusion proteins have been distinguished (36). Proteins containing HR regions and an N-terminal or N-proximal fusion peptide are classified as class I viral fusion proteins. Class II viral fusion proteins (e.g., the alphavirus E1 and the flavivirus E fusion protein) lack HR regions and have an internal fusion peptide. Their fusion protein is folded in tight association with a second protein as a heterodimer. Here, fusion activation takes place upon cleavage of the second protein.

The coronavirus fusion protein (S) shares several features with class I virus fusion proteins. It is a type I membrane protein, synthesized in the ER, and is transported to the plasma membrane. It contains two heptad repeat sequences, one located downstream of the fusion peptide and one in close proximity to the transmembrane region.

However, despite its similarity to class I fusion proteins, there are several characteristics that make the coronavirus S protein exceptional. One is the absence of an N-terminal or even N-proximal fusion peptide in the membrane-anchored subunit. Another peculiarity is the relatively large sizes of the HR regions (~100 and ~40 a.a.). Third, cleavage of the S protein is not required for membrane fusion; rather, it does not occur at all in the

group 1 coronaviruses. For these reasons, it is not likely to assume that coronavirus fusion protein is a class 1 fusion protein.

Heptad repeat regions play an important role in viral membrane fusion. Fusion proteins from widely disparate virus families have been shown to contain two such regions, one located close to the fusion peptide, the other generally in the vicinity of the viral membrane ((7); summarized in FIG. 8). Distances between the HR regions vary greatly, from some 50 a.a. as in HIV-1 to about 300 residues in *Spodoptera exigua* multicapsid nucleopolyhedrosis virus (71). The crystal structures resolved for influenza HA (4, 10, 75) HIV-1 and SIV gp41 (5, 8, 41, 63, 69, 76), MuMLV gp21 (19), Ebola virus GP2 (42, 68), HTLV-1 gp21 (32), Visna virus TM, (43), SV5 F1 (1), HRSV F1 (80) and NDV F (13) all show a central trimeric coiled coil constituted by three HR1 regions. In some of these structures (e.g., HIV-1 and SIV gp41, SV5 F1, Ebola virus gp2, Visna virus TM and HRSV F1) a second layer of helices or elongated peptide chains was observed contributed by HR2 domains which were packed in an anti-parallel manner into the hydrophobic grooves of the HR1 coiled coil, forming a six-helix bundle. In the full-length protein, such a conformation brings the fusion peptide present at the N-terminus of HR1 close to the transmembrane region that occurs at the C-terminal of HR2. With the fusion peptide inserted in the cellular membrane and the transmembrane region anchored in the viral membrane, such a hairpin-like structure facilitates the close apposition of cellular and viral membrane and enables subsequent membrane fusion (reviewed in (18)). Combined with the findings that peptides derived from these HR domains can act as potent inhibitors of fusion (reviewed in (18)), the biological relevance of the heptad repeat regions in the viral life cycle is obvious. Our studies of the heptad repeat motifs in coronavirus spike protein presented here show that coronaviruses use coiled coil formation for membrane fusion and cell entry mechanisms comparable to some other viruses, probably allowing coronavirus spike proteins to be classified as class I viral fusion proteins (36).

The coronavirus (MHV-A59) derived HR peptides exhibited a number of typical class I characteristics. First of all, the purified HR1 and HR2 peptides assembled spontaneously into unique, homogeneous multimeric complexes. These complexes were highly stable surviving, for instance, high concentrations (2%) of SDS and high temperatures (70-80°C). The peptides apparently associate with great specificity into an energetically very favorable structure.

Another typical feature was the observed secondary structure in the peptides. The CD spectra of both the individual and the complexed HR1 and HR2 peptides showed patterns characteristic of alpha-helical structure. Alpha-helix contents were calculated to be about 89% for the separate peptides and about 82% for their equimolar mixture. Consistent with these observations, the HR complex revealed a rod-like structure when examined by electron microscopy. The length of this structure (~14.5 nm) correlates well with the length predicted for an alpha-helix the size of HR1 (96 a.a.). Similar rod-like structures have been observed for other class I virus fusion proteins such as the influenza virus HA protein (12, 53), portions of the HIV-1 gp41 protein (70), and the Ebola virus GP2 protein (67) but the length of the MHV-A59 derived structures is substantially larger. This is presumably even more so for type I coronaviruses which have an insertion of two heptad repeats (14 a.a.; see FIG. 1) in both HR regions. These insertions into otherwise conserved areas suggest these additional sequences to associate with each other in the HR1-HR2 complex thereby extending the alpha-helical complex by exactly four turns. The significance of the exceptional lengths of coronavirus HR complexes may be that the higher energy gain of their formation corresponds with higher energy requirements for membrane fusion by these viruses.

Another important characteristic of class I viral fusion proteins is the formation of a heterotrimeric six-helix bundle during the membrane fusion process, resulting in a close allocation of the fusion peptide and the transmembrane domain. Consistently, protein dissection studies using proteinase K demonstrated an anti-parallel organization of the HR1 and HR2 alpha-helical peptides in the MHV-A59 HR complex. So far, no fusion peptides have been identified in any coronavirus spike protein but predictions for MHV S have located such fusion sequences at (7) or in (40) the N-terminus of HR1. In both cases an anti-parallel orientation of the HR1 and HR2 alpha helices ensures that the fusion peptide is brought into close proximity to the transmembrane region. Sequence analysis reveals that the 'e' and 'g' positions in the HR1 regions of all coronaviruses are primarily occupied by hydrophobic residues, unlike the 'e' and 'g' positions in the HR2 regions, which are mostly polar (see FIG. 1). The HR2 region also contains a strictly conserved N-linked glycosylation sequence, indicating its surface accessibility. Preliminary X-ray data on the HR1-HR2 complex show a six-helix bundle structure in the electron dense region (Bosch, B.J., Rottier, P.J.M, and Rey

F.A., unpublished results). The combined observations suggest a packing analogous to the fusion proteins of other class I viruses (e.g., HIV, SV5), where the HR1 and HR2 peptides can form a six-helix bundle with the long HR1 peptide centered in the middle as a three-stranded coiled-coil with the hydrophobic 'a' and 'd' residues in its inner core. The shorter HR2 peptide packs with its apolar interface in the hydrophobic grooves of the HR1 coiled coil, which expose the mostly hydrophobic residues on 'e' and 'g' positions.

Peptides derived from the heptad repeat regions of retrovirus (28, 30, 38, 47, 49, 58, 72, 73) and paramyxovirus (29, 35, 51, 77, 79) fusion proteins have been shown to strongly interfere with the fusion activity of these proteins. We observed the same effect when we tested the HR2 peptide of the MHV-A59 spike protein. Using a recombinant luciferase-expressing MHV-A59 the peptide acted as an effective inhibitor of virus entry at micromolar concentrations. Cell-cell fusion inhibition was even more efficiently blocked by the peptide as tested in a cell fusion luciferase assay system. However, peptides derived from the HR1 region had no or only a minor effect on virus entry and syncytia formation. HIV-1 gp41 derived HR peptides that inhibit membrane fusion have been shown not to bind to the native protein or to the six-helix bundle. They can only bind to an intermediate stage of gp41 occurring during the fusion process (9, 20, 31). Repeated passage of HIV in the presence of the inhibitory peptide DP178, which is derived from the C-terminal gp41 HR region, resulted in resistant viruses containing mutations in the N-terminal HR region (52). Inhibition of membrane fusion by the MHV HR2 peptide most likely takes place during an intermediate stage of the fusion process by binding of the peptide to the HR1 region in the spike protein. This binding, which may occur before, during or after the association of the HR1 regions into the inner trimeric coiled coil, presumably inhibits the subsequent interaction with native HR2 and, consequently, membrane fusion. For the HIV-1 gp41 and SV5 F protein also peptides corresponding to the HR1 region show membrane fusion inhibition, supposedly by binding to the native HR2 region (29, 72). It has been reported previously for HIV-1 that the HR1 peptide aggregates in solution (38) and that its inhibitory activity could be enhanced by fusing it to a designed soluble trimeric coiled coil, making the HR1 peptide more soluble (17). The MHV-A59 HR1 peptide is soluble in water but appeared to precipitate in salt solutions (data not shown). This solubility feature may have obscured the inhibitory potency of our HR1 derived peptides and

accounts for the negative results with these peptides in our fusion assays. The HR2 peptide (as well as, soluble forms of HR1) provides powerful antivirals for the therapy of coronavirus induced diseases both in animals and man.

Membrane fusion mediated by class I fusion proteins is accompanied by dramatic structural rearrangements within the viral polypeptide complexes (18). Though little is known of the coronavirus membrane fusion process (for a review, see (22)), the occurrence of conformational changes induced by various conditions has been described for MHV spikes (45). While MHV-A59 is quite stable at mildly acidic pH it is rapidly and irreversibly inactivated at pH 8.0 and 37°C (60). Under these conditions the S1 subunit dissociates from the virions and the S2 subunit aggregates concomitantly resulting in the aggregation of the particles. Due to the structural rearrangements in the spike, virions can bind to liposomes and the S2 protein becomes sensitive to protease degradation (27). Similar conformational changes can apparently also be induced at pH 6.5 by the binding of spikes to the (soluble) MHV receptor (21, 27) as this interaction enhances liposome binding and protease sensitivity as well (27). Virion binding to liposomes is presumably caused by the exposure of hydrophobic protein surfaces or of the fusion peptide as a result of the conformational change. It appears that the structural rearrangements in the spikes, whether elicited by elevated pH or soluble receptor interaction, reflect the process that naturally gives rise to the fusion of viral and cellular membranes. Accordingly, cell-cell fusion induced by MHV-A59 was maximal at slightly basic pH (60).

A number of studies on the MHV spike protein have shown the importance of the HR regions in membrane fusion. Three codon mutations (Q1067H, Q1094H and L1114R) in or close to the HR1 region of the spike protein were found to be responsible for the low pH requirement for fusion of some MHV-JHM variants isolated from persistently infected cells (23). Analysis of soluble receptor-resistant variants of this virus also pointed to an important role in fusion activity of the HR1 region and suggested that it interacts somehow with the N-terminal domain (S1N330-III; a.a. 278-288) of the spike protein (44). In yet another MHV-JHM variant a great reduction in cell-cell fusion was attributed to the occurrence of two mutations in the spike protein one of which again located in the HR1 region (A1046V), the other (V870A) in a small non-conserved HR region (N helix) close to the S cleavage site (33).

Acidification resulted in a clear enhancement of fusion by this double mutant. It was speculated that the three predicted helical regions (N helix, HR1 and HR2) all collapse into a low-energy coiled-coil during the process of membrane fusion (33). Herein we provide evidence that the HR1 and HR2 regions indeed can form such a low-energy coiled coil. Studies with the MHV-A59 S protein showed that mutations introduced at 'a' and 'd' positions in an N-terminal part of the HR1 region, a fusion peptide candidate, severely affected cell-cell fusion ability (40). This effect was not due to defects in spike maturation or cell surface expression. Finally, also codon mutations in the HR2 region were found to significantly reduce cell-cell fusion (39). Though these mutant spike protein were apparently impaired in oligomerization their surface expression was hardly affected.

In conclusion, our structural and functional studies show that the coronavirus spike protein can be classified as a class I viral fusion protein. The protein has, however, several unusual features that set it apart. An important characteristic of all class I virus fusion proteins known so far, is the cleavage of the precursor by host cell proteases into a membrane-distal and a membrane-anchored subunit, an event essential for membrane fusion. Consequently, the hydrophobic fusion peptide is then located at or close to the newly generated N-terminus of the membrane anchored subunit, just preceding the HR1 region. In contrast, the MHV-A59 spike does not have a hydrophobic stretch of residues at the distal end of S2, but carries a fusion peptide internally at a location that has yet to be determined (7, 40). Unlike other class I fusion proteins cleavage of the S protein into S1 and S2 has been shown to enhance fusogenicity (25, 61) but not to be absolutely required (2, 26, 59, 61). Rather, spikes belonging to group 1 coronaviruses are not cleaved at all.

The invention is further explained by the use of the following illustrative examples.

Example 1

MATERIALS AND METHODS

Plasmid constructions. For the production of peptides corresponding to amino acid residues 953-1048 (HR1), 969-1048 (HR1a), 1003-1048 (HR1b), 969-1010 (HR1c) and 1216-1254 (HR2) of the MHV-A59 spike protein, PCR fragments were prepared using as a template the plasmid pTUMS which contains the MHV-A59 spike gene (64). Primers were designed (see Table 1) to introduce into the amplified fragment an upstream *Bam*HI site, a downstream *Eco*RI site as well as a stop codon preceding the *Eco*RI site. The fragments corresponding to a.a. 953-1048 and 1216-1254 were additionally provided with sequences specifying a factor Xa cleavage site immediately downstream the *Bam*HI site. Fragments were cloned into the *Bam*HI/*Eco*RI site of the pGEX-2T bacterial expression vector (Amersham Bioscience) in frame with the GST gene just downstream of the thrombin cleavage site.

To establish a cell-cell fusion inhibition assay, the firefly luciferase gene was cloned under a T7 promoter and an EMCV IRES. The luciferase gene containing fragment was excised from the pSP-*luc*+ vector (Promega) by digestion with *Nco*I and *Eco*RV, treated with Klenow, and ligated into the *Bam*HI-linearized, Klenow-blunted pTN3 vector (65) yielding the pTN3-*luc*+ reporter plasmid.

Bacterial protein expression and purification. Freshly transformed BL21 cells (Novagen) were grown in 2 x YT (yeast-tryptone) medium to log phase (OD600~1.0) and subsequently induced by adding IPTG (GibcoBRL) to a final concentration of 0.4 mM. Two hours later cells were pelleted, resuspended in 1/25 volume of 10 mM Tris (pH 8.0), 10 mM EDTA, 1 mM PMSF and sonicated on ice (5 times 2 min). Cell homogenates were centrifuged at 20,000 x *g* for 60 min at 4°C. To each 50 ml of supernatant 2 ml glutathione-sepharose 4B (Amersham Bioscience; 50% v/v in PBS) was added and incubated overnight (O/N) at 4°C under rotation. Beads were washed three times with 50 ml PBS and resuspended in a final volume of 1ml PBS. Peptides were cleaved from the GST moiety on the beads using 20 U of thrombin (Amersham Bioscience) by incubation for 4 h at room temperature (RT). Peptides in the supernatant were purified by high pressure reversed phase chromatography (RP-HPLC) using a Phenyl-5PW RP column (Tosoh) with a linear gradient of acetonitrile containing 0.1%

trifluoroacetic acid. Peptide containing fractions were vacuum-dried O/N and dissolved in water. Peptide concentration was determined by measuring the absorbance at 280 nm (24) and by BCA protein analysis (Micro BCA™ Assay Kit, Pierce).

Temperature stability of HR1-HR2 complex. An equimolar mix of peptides HR1 and HR2 (80 μM each) in H_2O was incubated at RT for 1 h. After addition of an equal volume of 2 x tricine sample buffer (0.125 M Tris pH 6.8, 4% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.004 g bromophenol blue) (55), the mixtures were either left at RT or heated for 5 min at different temperatures and subsequently analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in 15% tricine gel (55).

CD spectroscopy. CD spectra of peptides (25 μM in H_2O) were recorded at RT on a Jasco J-810 spectropolarimeter, using a 0.1 mm path length, 1 nm bandwidth, 1 nm resolution, 0.5 s response time and a scan speed of 50 nm/min. The alpha-helix content was calculated using the program CDNN (http://bioinformatik.biochemtech.uni-halle.de/cd_spec/).

Electron Microscopy. A preincubated equimolar mix of the peptides HR1 and HR2 was subjected to size-exclusion chromatography (Superdex™ 75 HR 10/30, Amersham Pharmacia Biotech). A sample from the HR1-HR2 peptide complex containing fraction was adsorbed onto a discharged carbon film, negatively stained with a 2% uranyl acetate solution and examined with a Philips CM200 microscope at 100 kV.

Proteinase K treatment. Stock solutions (1 mM) of the peptides HR1, HR1a, HR1b, HR1c and HR2 in water were diluted to 80 μM in PBS. Peptides on their own (80 μM) or after preincubation for 1 h at 37°C with HR2 (80 μM each) were subsequently subjected to proteinase K digestion (1% wt/wt, proteinase K/peptide) for 2 h at 4°C. Samples were immediately subjected to tricine SDS-PAGE analysis. Protease resistant fragments were also separated and purified by RP HPLC and characterized by mass spectrometry.

Virus-cell fusion assay. The potency of HR peptides in inhibiting viral infection was determined using a recombinant MHV-A59, MHV-EFLM that expresses the firefly luciferase gene (C.A.M. de Haan and P.J.M. Rottier, manuscript in preparation). LR7 cells (34) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; GIBCO BRL). LR7 cells grown in 96-wells plates were inoculated with MHV-EFLM in DMEM at a multiplicity of infection (MOI) of 5

in the presence of varying concentrations of peptide ranging from 0.4 – 50 μM. After 1 h, cells were washed with DMEM and medium was replaced with DMEM containing 10% FCS. At 5 h post infection (p.i.) cells were harvested in 50 μl 1x Passive Lysis buffer (Luciferase Assay System, Promega) according to the manufacturer's protocol. Upon mixing of 10 μl cell lysate with 40 μl substrate, luciferase activity was measured using a Wallac Betalumino meter.

Cell-cell fusion assay. $2x10^6$ LR7 cells, used as target cells, were washed with DMEM and overlaid with transfection medium consisting of 0.2 ml DMEM containing 10 μl of lipofectin (Life Technologies) and 4 μg of the plasmid pTN3-*luc*+. After 10 min at RT, 0.8 ml DMEM was added and incubation was continued at 37°C. BSR T7/5 cells - BHK cells constitutively expressing T7 RNA polymerase (3); a gift from Dr. K.K. Conzelmann - were grown in BHK-21 medium supplemented with 10% FCS, 100 IU of penicillin/ml and 1 mg/ml geneticin (GIBCO BRL). $1x10^4$ BSR T7/5 cells, designated as effector cells, were infected in 96-wells plates with wild-type vaccinia virus at an MOI of 1 in DMEM at 37°C. After 1 h, the cells were washed with DMEM and incubated for 3 h at 37°C with transfection medium consisting of 50 μl DMEM containing 1 μl lipofectin and 0.2 μg of the plasmid pTUMS (65), which carries the MHV-A59 spike gene under the control of a T7 promoter. Then, $3x10^4$ of target cells in 100μl DMEM were added and the cells were incubated for another 4 h in the presence or absence of HR peptide. Cells were lysed and luciferase activity was measured as mentioned above.

RESULTS

HR1 and HR2 regions in coronavirus spike proteins.

The S2 subunit ectodomain of coronaviruses contains two heptad repeat domains HR1 and HR2, which are conserved in sequence and position (15) (diagrammed in FIG. 1A). HR2 is located adjacent to the transmembrane domain while HR1 occurs at about 170 a.a. upstream of HR2. FIG. 1B shows a protein sequence alignment of the HR1 and HR2 regions for 6 coronaviruses from the three antigenic clusters. The sequence alignment reveals a remarkable insertion of exactly two heptad repeats (14 a.a.) in both the HR1 and the HR2 domain of the spike protein of the group 1 coronaviruses HCV-229E (human coronavirus strain 229E) and FIPV (feline infectious peritonitis virus strain 79-1146).. Another characteristic feature is that

the length of the linker region between the HR2 region and the transmembrane region is strictly conserved in all coronavirus spike proteins.

HR1 and HR2 can form an hetero-oligomeric complex.

To study the heptad repeat regions in the S2 subunit of MHV-A59, peptides corresponding to the heptad repeat residues 953-1048 (HR1), 969-1048 (HR1a), 969-1048 (HR1b), 969-1003 (HR1c) and 1216-1254 (HR2) (FIG. 1B) were produced in bacteria as GST fusion proteins. Peptides were affinity purified using glutathione-sepharose beads, proteolytically cleaved from the resin and purified to homogeneity by reversed-phase HPLC. Masses of the peptides, as determined by mass spectrometry, matched their predicted Mw (HR1, 10,873 Da; HR1a, 8,653 Da; HR1b, 5,631 Da; HR1c, 4,447 Da; and HR2, 5,254 Da). To study an interaction between the two HR regions, the purified peptides HR1 and HR2 were incubated alone (80 μM) or in an equimolar (80 μM each) mixture for 1 h at 37°C and the samples were subjected to SDS-PAGE either directly or after heating for 5 min at 95°C (FIG. 2A). While the peptides migrated according to their molecular weight after separate incubation, most of the protein of the preincubated mixture of HR1 and HR2 migrated as a higher molecular weight complex with a slightly lower mobility than the 29 kDa marker. Upon heating, the complex dissociated giving rise to the individual subunits HR1 and HR2. We also tested the other HR1 peptides for interaction with HR2. While we did not observe complexes upon mixing of HR2 with HR1b or HR1c (data not shown), a higher molecular weight species co migrating with the 29 kDa marker was found when HR1a was incubated with HR2 (FIG. 2B), though the extent of complex formation appeared to be lower than with peptide HR1. Higher molecular weight species were not seen. The results indicated that the HR1 region contains the information to associate with the HR2 region into a hetero-oligomeric complex and that this complex was stable in the presence of 2% SDS.

HR1-HR2 complex is highly temperature resistant.

Next, we determined the stability of the HR1-HR2 complex at increasing temperatures. An equimolar (80 μM each) mix of the two peptides was again incubated for 1 h at 37°C and subsequently heated for 5 minutes at different temperatures in 1x tricine sample buffer or left

at RT. The complexes were analyzed by SDS-PAGE in 15% gel. As FIG. 3 demonstrates, the high molecular weight complexes remained intact up to 70°C, dissociated partly at 80°C and fully at 90°C. The stability of the complex at high temperatures indicates that the peptides are held together by strong interaction forces in an energetically favorable conformation.

HR1, HR2 and the HR1-HR2 complex are highly α-helical.

The secondary structure of the HR peptides was examined by circular dichroism. The CD spectra of HR1, HR2 and of an equimolar mixture of HR1 and HR2 were recorded (FIG. 4). The spectra showed clear minima at 208 nm and 222 nm, which is characteristic of alpha-helical structure. Calculations revealed that the alpha-helical contents of the individual HR1 and HR2 peptides and of the mixture of the two peptides were 89.2%, 89.3% and 81.9%, respectively.

The HR1-HR2 complex has a rod-like structure.

The overall shape of the HR1-HR2 complex was examined by electron microscopy. Complexes were purified and viewed after negative staining. Electron micrographs revealed rod-like structures (FIG. 5). Based on measurements of 40 particles an average length of 14.5 nm (± 2 nm) was calculated. This length is consistent with an alpha-helix of approximately 90 a.a. in length, which corresponds approximately to the predicted length of the HR1 coiled coil region. Similar rod-shaped complexes have been reported for the influenza virus HA protein (12, 53), for portions of the HIV-1 gp41 protein (70) and for the Ebola virus GP2 protein (67).

HR1 and HR2 helices associate in an anti-parallel manner.

The relative orientation and position of HR2 with respect to HR1 in the complex was examined by limited proteolysis using proteinase K in combination with mass spectrometry. Complexes were generated by incubation of the HR2 peptide with each of peptides HR1, HR1a, HR1b and HR1c. The reaction mixtures as well as the individual peptides were then treated with proteinase K. Samples from each reaction were analyzed by tricine SDS-PAGE (data not shown). Using RP HPLC the protease resistant fragments were purified and their molecular weight (MW) was determined by mass spectrometry, which allowed us to identify

the protease resistant cores of the peptides. For each protease resistant core a unique amino acid composition could be deduced that allowed the unequivocal identification of the peptides in the different samples. FIG. 6 gives a schematic overview of the proteinase K resistant fragments. Digestion of HR1 alone left a protease-resistant fragment with a MW of 6,801 Da corresponding to residues 976-1040. Although CD spectra had indicated a folded structure, HR2 was completely degraded by proteinase K. However, in the presence of HR1 HR2 was fully protected from proteolytic degradation. HR2 was able to rescue 18 additional residues at the N terminus of HR1, leaving a fragment of 8,675 Da corresponding to residues 958-1040.

Proteolysis of the HR1a peptide alone generated the same fragment (residues 976-1040) as obtained with HR1. In the HR1a-HR2 mixture, the HR2 peptide was completely protected against degradation by HR1a, while HR2 fully shielded the N-terminus of HR1a for proteolysis, including the glycine and serine residues originating from the thrombin cleavage site.

Although a higher molecular weight species could not be detected by tricine SDS-PAGE (data not shown), the protease treatment of the HR1c-HR2 complex left a protease resistant core. HR1c was fully sensitive for proteinase K, but was completely protected in the presence of HR2. HR2 itself was partly protected against proteolysis by HR1c, yielding a fragment of 3,583 Da that represents residues 1225-1254. Importantly, this HR2 fragment has an intact C-terminus but is degraded at its N-terminus. HR1c has the same N-terminus as HR1a but is truncated at its C-terminus. Thus, its inability to protect the HR2 N-terminus combined with the full protection provided by HR1a implies an anti-parallel association of the HR1 and HR2 helices in the hetero-oligomeric complex. The peptide HR1b was fully sensitive to proteinase K both by itself and when mixed with HR2. HR1b could not prevent proteolysis of HR2 either. Altogether, the proteolysis results suggest the anti-parallel association of HR2 and HR1 to occur in the middle part of HR1.

HR2 strongly inhibits viral entry and syncytium formation.

The formation of stable HR complexes is supposedly an essential step in the process of membrane fusion during viral cell entry. Thus, we evaluated the potency of our HR peptides in inhibiting MHV entry making use of a recombinant MHV-A59, MHV-EFLM that expresses

the firefly luciferase reporter gene. Cells were inoculated with MHV-EFLM in the presence of different concentrations of the peptides HR1, HR1a, HR1b, HR1c and HR2. After 1 h, the cells were washed and culture medium without peptide was added. At 4 h p.i., i.e. before syncytium formation takes place, cells were lysed and tested for luciferase activity (FIG. 7A). HR1, HR1a and HR1b were not able to inhibit virus entry up to concentrations of 50 μM. In contrast, HR2 blocked viral entry in a concentration-dependent manner inhibition being almost complete at a concentration of 50 μM.

We also studied the ability of the HR peptides in blocking cell-cell fusion. To this end we established a sensitive fusion assay based on the co-culturing of BHK cells expressing the bacteriophage T7 polymerase as well as the MHV-A59 spike protein, with murine L cells transfected with a plasmid carrying a luciferase gene cloned behind a T7 promoter. Fusion of the cells was determined by measuring luciferase activity. The effects of adding the HR peptides during the co-culturing of the cells are compiled in FIG. 7B. The HR2 peptide again appeared to be a potent inhibitor able to efficiently block cell-cell fusion. A 1000x reduction in luciferase activity was measured at a concentration of 10 μM, whereas essentially no activity was observed at a concentration of 50 μM. Of the HR1 peptides only the HR1b peptide had a minor effect at the highest concentration of 50 μM.

Example 2

Inhibition of cell-cell fusion after FIPV infection

FCWF cells were infected with FIPV strain 79-1146 with an moi of 1. 1 hour after infection the cells were washed and medium was replaced by medium containing the GST-FIPV fusion proteins at different concentrations. 8 hours after infection, cells were fixed and scored for syncytia formation (see, Table 2). The amino acid sequence of HR1 and HR2 of FIP is shown in FIG.9

Example 3

Inhibition of SARS-CoV infection of Vero cells by peptides derived from the HR1 and/or HR2 region of SARS-CoV.

Material and methods

Plasmid constructions. For the production of peptides corresponding to the HR1 and HR2 regions of the SARS-CoV spike protein, PCR fragments were prepared using as a template a SARS-CoV (strain 5688, Kuiken) cDNA covering the S gene. Primers were designed (see Table 3) to introduce into the amplified fragment an upstream *Bam*HI site, a downstream *Eco*RI site as well as a stop codon preceding the *Eco*RI site. Fragments were cloned into the *Bam*HI/*Eco*RI site of the pGEX-2T bacterial expression vector (Amersham Bioscience) in frame with the GST gene just downstream of the thrombin cleavage site. For the production of HR peptides with an N-terminal hydrophilic FLAG-tag (DYKDDDDK) a primer dimer (Table 3) containing the FLAG-tag encoding sequence was cloned into the *Bam*HI site of the pGEX-2T vector, thereby knocking out the 5' *Bam*HI site. The resulting vector was used to clone the HR1 and HR2 PCR products of SARS-CoV spike gene into the *Bam*HI/*Eco*RI site.

Bacterial protein expression and purification. Freshly transformed BL21 cells (Novagen) were grown in 2 x YT (yeast-tryptone) medium to log phase (OD600 ~1.0) and subsequently induced by adding IPTG (GibcoBRL) to a final concentration of 0.4 mM. Two hours later cells were pelleted, resuspended in 1/25 volume of 10 mM Tris (pH 8.0), 10 mM EDTA, 1 mM PMSF, and sonicated on ice (5 times 2 min). Cell homogenates were centrifuged at 20,000 x *g* for 60 min at 4°C. To each 50 ml of supernatant 2 ml glutathione-sepharose 4B (Amersham Bioscience; 50% v/v in PBS) was added and the suspensions were incubated overnight (O/N) at 4°C under rotation. Beads were washed three times with 50 ml PBS and resuspended in a final volume of 1ml PBS. Peptides were cleaved from the GST moiety on the beads using 20 U of thrombin (Amersham Bioscience) by incubation for 4 h at RT. Peptides in the supernatant were purified by reversed phase high pressure liquid chromatography (RP HPLC) using a Phenyl-5PW RP column (Tosoh) with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. Peptide containing fractions were vacuum-dried O/N and dissolved in water. Peptide concentrations were determined by measuring the

absorbance at 280 nm (Gill and von Hippel 1989) and by BCA protein analysis (Micro BCA™ Assay Kit, Pierce).

Inhibition of SARS-CoV infection.

Vero 118 cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Biowhittaker, Belgium) supplemented with 5% fetal bovine serum (FBS; Greiner), penicillin (100 U/ml), streptomycin (100 µg/ml), and 2 mM L-glutamine. The initial experiments were performed on Vero 118 cells grown on cover slips in 24 well plates ($2x10^5$ cells/ well) at 37°C. Cells were inoculated (MOI = 0.5) in the presence of HR peptide at different concentration (25, 5 and 0 µM). After 1 h, the inoculum was removed, the cells were washed twice with IMDM and the cells were overlaid with IMDM containing 5% FBS and the peptide at similar concentration as used in the inoculum. After O/N incubation, plates were washed twice with PBS and fixed by 4% formaldehyde for 15 min and 70% ethanol plus 0,5% H_2O_2 for 15 min at RT After washing the plates twice with PBS + 0.5% Tween-20 and twice with PBS, the cover slips were incubated with a human polyclonal reconvalescent serum(1: 50) for 1 hr at 37°C. FITC labeled anti human serum was used as a conjugate in a 1:300 dilution. Pictures of FITC fluorescent cells were taken using a Olympus camera mounted on a Leitz microscope.

The second set of inhibition experiments was performed on Vero 118 cells in 96 well plates (10^4 cells/ well). Cells were infected in triplicate with 100 $TCID_{50}$ of SARS-CoV (strain 5688, fourth passage) in the presence of various peptide concentrations, ranging from 0.4 µM to 50 µM, for 1 h at 37°C in a CO_2-incubator. Cells were then washed twice with IMDM and the medium was replaced with IMDM containing 5% FBS. After incubation for 9 h, plates were washed twice with PBS and fixed by 4% formaldehyde for 15 min and 70% ethanol plus 0.5% H_2O_2 for 15 min at RT. After washing the plates twice with PBS + 0.5% Tween-20 and twice with PBS, the fixed and permeabilized cells were incubated with a ferret polyclonal antiserum (1:40) for 1 hr at 37°C. Horse radish peroxidase (HRP) labeled goat-anti-ferret antibodies (DAKO, USA) were used as a conjugate in a 1:50 dilution. Reaction was developed with 3-amino-9-ethylcarbazole (AEC; Sigma, Zwijndrecht) according to the manufacturer's

instructions. SARS-CoV positive cells were counted using the light microscope and the effective peptide concentration at which 50% of the infection was inhibited (EC_{50}) was determined. Inhibition of MHV by HR peptides was tested as described above but using LR7 cells (Kuo, Godeke et al. 2000) rather then VERO 118 cells. IPOX detection of MHV positive cells was carried out by using a rabbit polyclonal antibody against MHV (1:300) (Rottier, Armstrong et al. 1985) in combination with a HRP swine-anti rabbit antibody (1:300) (DAKO, USA). Experiments were performed in triplicate, and carried out in duplicate.

Temperature stability of SARS-CoV and MHV HR1-HR2 complex. Equimolar mixes of HR1 and HR2 peptides (100 μM each) of SARS-CoV and MHV were incubated in parallel at RT for 3 h, to allow HR1-HR2 complex formation. 25 μl of each mix was pooled and an equal volume of 2 x Tricine sample buffer (0.125 M Tris pH 6.8, 4% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.004 g bromophenol blue) (55)was added. The mixtures were either left at RT or heated for 5 min at different temperatures and subsequently analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in 15% Tricine gel (55)

CD spectroscopy. CD spectra of the HR1 and HR2 peptides (20 μM in H_2O) or a preincubated equimolar mix of HR1 and HR2 (20 μM each in H_2O) were recorded at RT on a Jasco J-810 spectropolarimeter, using a 0.1 mm path length, 1 nm bandwidth, 1 nm resolution, 0.5 s response time and a scan speed of 50 nm/min. The alpha-helical content of the peptides was calculated using the program k2d (http://www.embl-heidelberg.de/~andrade/k2d/).

Proteinase K treatment. Stock solutions (250 μM) of the peptides HR1a, HR1c and HR2 in water were diluted to 100 μM in 50 mM Tris pH 7.0. Peptides on their own (100 μM) or HR1-HR2 mixtures (100 μM each) preincubated for 3 h at 37°C were subjected to proteinase K digestion (1% wt/wt, proteinase K/peptide) for 2 h at 4°C. Protease resistant fragments were separated and purified by RP HPLC and characterized by mass spectrometry.

Results

HR regions in the SARS-CoV spike protein

As shown by the alignment in FIG. 1, two heptad repeat (HR) regions are present in the C-terminal S2 domain of the SARS-CoV spike protein as they were detected before in other coronavirus spike proteins (15). One region (HR2) is located adjacent to the transmembrane domain, the other (HR1) some 170 residues upstream. In all coronaviruses, HR1 is consistently larger than HR2. However, the group 1 coronaviruses show a remarkable insertion of two heptad repeats (14 aa) in both HR regions. This insertion is lacking in the SARS-CoV HR regions. The HR2 region of SARS-CoV contains three conserved N-glycosylation sites (N-X-S/T; FIG. 1B).

HR peptides and their infection inhibitory activities

Peptides corresponding to the HR regions were expressed using the bacterial GST expression and purification system. They were purified to homogeneity using RP HPLC and their molecular masses were verified by mass spectrometry. Peptides were subsequently tested for their inhibitory potency in an infection inhibition assay. VERO cells were inoculated with SARS-CoV (MOI 0.5) in the absence or presence of different concentrations of a particular peptide and the extent of infection was evaluated using an indirect immunofluorescence assay. As shown in FIG. 11A, for one of the initial peptides tested, HR2-1, a clear concentration dependent inhibition of SARS-CoV infection could be observed. This effect was sequence specific as no inhibition was seen with a corresponding peptide derived from the HR2 region of MHV (mHR2), known to block MHV infection.

To study the sequence dependence and to optimize the efficacy of the inhibition we prepared two sets of peptides the sequences of which are compiled in FIG. 10B. One set consisted of HR2-1 based peptides: a series of peptides with increasing 4-residue N-terminal truncations (HR2-2 – HR2-7), one peptide with a 4-residue C-terminal extension (HR2-8) and two peptides with 4- and 8-residue C-terminal truncations (HR2-9 and HR2-10, respectively). The other set consisted of peptides corresponding to the HR1 region, with peptide HR1 comprising almost the entire heptad repeat region and peptides HR1a-c representing N-terminal and C-terminal truncations thereof. These peptides were tested similarly, but the infection levels were now determined in a technically different format using immune

peroxidase staining followed by an automated read-out of the percentage of infected cells. FIG. 10B shows the EC_{50} values obtained, i.e. the concentrations calculated to cause a 50% reduction of infection. It is clear that only slight truncations at either side of the HR2-1 peptide are tolerated without loss of inhibitory activity. Actually, shortening HR2-1 just by 4 residues at the N-terminal (HR2-2) or the C-terminal side (HR2-9) resulted in significantly enhanced inhibition. The most effective peptide of the panel was HR2-8, which carried the C-terminal 4-residue extension. It had an EC_{50} value of 17 μM. The inhibition efficiency of this peptide was clearly lower than that of an HR2 peptide of MHV, mHR2, which had an EC_{50} value of 0.9 μM when tested in the MHV infection system. Of the panel of HR1 derived peptides none showed any measurable inhibitory effect on SARS-CoV infection under the conditions used in this experiment..

HR1-HR2 complex formation

We have previously shown by Tricine SDS-PAGE analysis that the HR1 and HR2 peptides of the MHV S protein, when mixed together, assemble into an oligomeric complex that is resistant to 2% SDS, the SDS concentration used in this analysis. By the same approach we observed that the HR1 and HR2 peptides of the SARS-CoV spike protein behave likewise. As shown in FIG. 12A for equimolar mixtures of similar HR peptides from both viruses, SDS-stable oligomeric complexes are formed that dissociate upon heating.

These observations do not necessarily imply that the complexes are composed of both HR peptides: in the mixture one peptide might simply catalyze the homomultimerization of the other. To confirm the presence of both HR1 and HR2 in the complex, FLAG-tagged HR peptides were prepared in which the polar FLAG octapeptide (DYKDDDDK (SEQ ID NO:__)) was appended to the N-termini of HR1 (FLAG-HR1) and HR2 (FLAG-HR2). Preincubated mixtures of HR1+HR2, FLAG-HR1+HR2, HR1+FLAG-HR2 and FLAG-HR1+FLAG-HR2 were analyzed in 15% Tricine SDS-PAG together with the individual peptides (FIG. 12B). The individual FLAG-tagged HR peptides migrated slower in the gel than their non-tagged homologues. All combinations of HR1 and HR2 peptide produced the higher molecular weight band, indicating that the addition of the FLAG tag did not prevent complex formation. The combination of FLAG-HR1+HR2 and of HR1+FLAG-HR2 each

produced a complex that had lower gel mobility than the non-tagged HR1+HR2 complex. Combining the two tagged peptides resulted in an additional mobility decrease. These observations imply that both the HR1 and the HR2 peptide are present in the complex.

Stoichiometry of peptides in the HR1-HR2 complex

The availability of the FLAG-tagged HR peptides provided us with the means to determine the stoichiometry of the peptides in the HR complex. As the FLAG-tag did not interfere with complex formation its distinctive effect on the electrophoretic mobility of the tagged peptides was exploited to determine the number of HR1 and HR2 peptides in the complex. FLAG-tagged and non-tagged HR2 peptides were mixed in different ratios and subsequently incubated for 3 h at room temperature (RT) with equimolar amounts of HR1 peptide to allow complex formation. Subsequent SDS-PAGE analysis revealed four bands when the HR1 peptide had been incubated with a 1:1 mixture of FLAG-tagged and non-tagged HR2 peptides (FIG. 13-I). The fastest migrating band co migrated with the complex obtained with non-tagged HR2 peptide only, while the band with the lowest mobility corresponded to the complex obtained with the FLAG-tagged HR2 peptide. Consequently, the two intermediate bands represent complexes containing one and two FLAG-tagged HR2 peptides, respectively. Note that the relative intensities of the four bands correspond well with the predicted ratio of formation of the different complexes (1/8, 3/8, 3/8, 1/8 respectively), calculated under the assumption that the tag is fully inert.

The reciprocal approach was used to determine the number of HR1 peptides in the complex. In this case FLAG-tagged and non-tagged HR1 peptides were combined with non-tagged HR2 peptide. However, when a 1:1 mixture of the two HR1 forms was incubated with HR2, only two bands were observed in the gel (FIG. 13-II), the faster one comigrating with the HR1-HR2 complex, the slower one corresponding with the FLAG-HR1-HR2 complex. One interpretation of this result is that the complex contains just one HR1 peptide molecule. Alternatively, HR1 peptides in solution assemble into homo-oligomers already in the absence of HR2. These oligomers are sufficiently stable to prevent the exchange of peptides when tagged and non-tagged HR1 complexes are mixed and, as a result, such a mixture will yield only two forms of hetero-oligomeric complexes upon addition of HR2. In view of this latter

possibility we repeated the experiment after we had first denatured the putative HR1 oligomers. Thus, acetonitrile – an anorganic solvent - was added to solutions of HR1 and FLAG-tagged HR1 to a concentration of 50% (v/v). The solutions were mixed, briefly incubated after which the acetonitrile was removed by evaporation. Equimolar mixtures were again prepared of the different HR1 forms and HR2, which were incubated and finally analyzed by Tricine SDS-PAGE. As FIG. 13-III reveals, we now observed four bands in the sample containing both tagged and non-tagged HR1, indicating the presence of three HR1 peptides in the complex. The combined results are consistent with HR1 and HR2 forming a hexameric complex composed of three molecules HR1 and HR2 each.

Temperature stability of HR1-HR2 complex

The stability of the SARS-CoV HR1-HR2 complex to temperature dissociation was assessed in comparison to that of the corresponding MHV complex. Equal amounts of both complexes were combined and the solution was adjusted to 1x Tricine sample buffer. Equal samples were taken, incubated in parallel for 5 min at different temperatures and subsequently analyzed by 10% Tricine SDS-PAGE (FIG. 14). Due to their distinct electrophoretic mobilities, the SARS-CoV and MHV complexes could clearly be distinguished allowing the direct comparison of their temperature sensitivity. Surprisingly, the SARS-CoV HR complex appeared to be significantly less stable (dissociated at 70°C) than the MHV complex (dissociated at 90°C).

Secondary structure of HR1 and HR2 peptides and of HR1-HR2 complex

Circular dichroism (CD) was used to determine the secondary structure of the individual peptides HR1 and HR2 and of the HR1-HR2 complex. The CD spectra show that the peptides have a high alpha-helicity both on their own and in the complex (FIG. 15). The calculated values of the helical content were 85% (HR1), 81% (HR2) and 88% (HR1-HR2).

Limited proteolysis on HR1-HR2 complex

Strongly folded protein structures are often resistant to proteolytic degradation. To obtain structural information about the HR1-HR2 complex we carried out limited digestions

with proteinase K, purified the resistant fragments by RP HPLC (FIG. 16, upper part) and analyzed the fragments by mass spectrometry (FIG. 16, lower part). For the individual peptides the results showed that HR2 was completely degraded by the enzyme while of the HR1a peptide only the C-terminal 6 residues were sensitive to proteinase K, indicating a strong folding of this latter peptide. When a mixture of the two peptides was analyzed the HR2 peptide was entirely protected from proteolytic breakdown. A similar analysis carried out with a C-terminally truncated version of HR1a, HR1c, revealed that now the N-terminus of HR2 was no longer protected. These results indicate that in the HR1-HR2 complex, the HR1 and HR2 helices are oriented in an anti-parallel fashion.

Our functional and biochemical analyses of the SARS-CoV spike HR regions shows that the virus makes use of a membrane fusion mechanism that has similarities with the fusion mechanism of class I fusion proteins, in which the HR regions play a prominent role. We show that peptides corresponding to the HR2 domain, but not those derived of the HR1 domain of the SARS-CoV spike protein can inhibit virus infection. We show here that HR2 peptides are able to bind stably to HR1 peptides, as has been observed previously for coronavirus, retrovirus and paramyxovirus fusion proteins. Analogous to the HIV-1 gp41, SV5 F and HRSV F proteins (69, 8, 1, 80, the HR1-HR2 complex was found to consist of a six-helix bundle that is composed of three HR1 and three HR2 alpha-helical peptides. The high resistance of the HR1 peptide to proteinase K, the inability of separately preincubated FLAG-tagged and non-tagged HR1 peptides to form mixed hexamers unless first dissociated by acetonitrile, and the highly alpha-helical character of the peptide are all observations suggesting that SARS-CoV HR1, in the absence of HR2, already forms a (trimeric) coiled coil. The proteolysis data point to an anti-parallel packing of HR2 with respect to HR1, presumably through interaction of the hydrophobic interface of the HR2 helix with the hydrophobic groove in the HR1 coiled coil created by the - mostly hydrophobic - e and g residues of HR1. Formation of such an anti-parallel six-helix bundle has been shown to be essential in the membrane fusion process, by pulling the viral and cellular membrane together. In the full-length spike protein such a structure brings the fusion peptide - N-terminal of HR1 - in close proximity to the transmembrane domain - C-terminal of HR2 – thereby enabling membrane fusion. The infection inhibiting effect of peptides corresponding to HR2 can be

explained by their competitive binding to the HR1 region of the SARS-CoV spike protein, which prevents formation of the six-helix bundle and, consequently, membrane fusion

The HR2-8 peptide can be used as a lead for the development of more effective SARS-CoV peptide inhibitors. Alternatively, the HR peptides might be used as a vaccine, since antibodies directed against the HR2 peptides of HIV-1 inhibit virus infection. Hence, the HR peptides provide a basis for therapeutic and/or prophylactic agents against SARS-CoV as well as against other coronaviruses.

Example 4

The coronavirus fusion peptide

Transmembrane prediction using the TMAP program

The TMAP program www.mbb.ki.se/tmap/index.html was used to predict transmembrane segments in coronavirus spike proteins using multiple sequence alignments. A Clustal W alignment was used of spike protein sequences from nine coronaviruses including FIPV (feline infectious peritonitis virus, strain 79-1146; VGIH79), TGEV (porcine transmissible gastroenteritis virus, strain Purdue; P07946), PEDV (porcine epidemic diarrhea virus; NP_598310), HCoV-229E (human coronavirus, strain 229E; VGIHHC), BCoV (bovine coronavirus, strain F15; P25190), MHV (mouse hepatitis virus, strain A59; P11224), HCoV-OC43 (human coronavirus, strain OC43; CAA83661), SARS-CoV (strain TOR2; P59594), and IBV (infectious bronchitis virus, strain Beaudette; P11223).

The TMAP program, designed to identify transmembrane domains in proteins, was used in search for the coronavirus fusion peptide. Nine coronaviral spike protein sequences (FIG. 17, lower part) were used for the Clustal W alignment on which the prediction by the TMAP program is based. Three hydrophobic regions were identified (FIG. 17, middle part). Two of these, i.e. the regions in the N- and C-terminal part of the protein, represent the well-known signal sequence and transmembrane anchor, respectively. The third domain is found immediately upstream of HR1. This location combined with its hydrophobicity and the presence of a conserved proline in it are characteristics indicating that this domain functions as the coronavirus fusion peptide.

The identity of the fusion peptide in the coronavirus spike protein has not yet been established. Generally, class I fusion proteins require cleavage for fusion activation. As a result the fusion peptide ends up at or close to the N-terminus of the membrane-anchored subunit. Unlike other class I fusion proteins, coronavirus spike proteins lack the cleavage requirement for virus infectivity. Cleavage inhibition of the MHV spike protein by a furin blocker does not affect virus infectivity, rather, group 1 coronaviruses are not cleaved at all. We could not observe any significant cleavage of the expressed spike protein. Additionally, the cleavable coronavirus spike proteins lack a hydrophobic region adjacent to the cleavage site. This suggests that coronavirus spike proteins use an internal fusion peptide like the VSV G protein and class II fusion proteins, such as the TBEV E and SFV E1 fusion proteins. In order to predict the location of the fusion peptide, we have used a transmembrane prediction program TMAP], which predicts transmembrane domains (TM) in protein sequences using multiple alignments. In the Clustal W alignment of all known coronavirus spike sequences, the TMAP program predicted three TM domains (FIG. 17). One represented the signal sequence (SARS-CoV-S residues 1 - 15), the other the transmembrane anchor (residues 1195 - 1223), and the third hydrophobic region was predicted immediately N-terminal of the HR1 region (residues 858 - 886). Careful inspection of this region reveals that it has fusion peptide characteristics like a high alanine and glycine content and a conserved proline residue (residue 879), which is characteristic of internal fusion peptides. This region was previously recognized by Chambers and coworkers (7) as a potential fusion peptide for coronaviruses. The formation of the anti-parallel six-helix bundle during the fusion reaction brings this fusion peptide in close proximity to the transmembrane anchor of the full-length protein, which results in the merging of viral and cellular membranes.

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Table 1. Primers used for PCR of HR regions

Primer	*Polarity*	*Sequence (5'- 3')*	*HR product*
973	+	GTGGATCCATCGAAGGTCGTCAATATAGA ATTAATGGTTTAG (SEQ ID NO:__)	HR1
974	+	GTGGATCCATCGAAGGTCGTAATGCAAAT GCTGAAGC (SEQ ID NO:__)	HR1b
975	-	GGAATTCAATTAATAAGACGATCTATCTG (SEQ ID NO:__)	HR1, HR1a, HR1b
976	-	CGAATTCATTCCTTGAGGTTGATGTAG (SEQ ID NO:__)	HR2
990	+	GCGGATCCATCGAAGGTCGTGATTTATCTC TCGATTTC (SEQ ID NO:__)	HR2
1151	+	GTGGATCCAACCAAAAGATGATTGC (SEQ ID NO:__)	HR1a, HR1c
1152	-	GGAATTCAATTGAGTGCTTCAGCATTTG (SEQ ID NO:__)	HR1c

Table 2

Inhibition of cell-to-cell fusion

FCFW cells/FIPV infected		
	GST-HR1	**GST-HR2**
10 ng	+++	-
1 ng	+++	+
0.1ng	+++	++
0 ng	+++	+++

Syncytia formation +++

Table 3. Primers used for PCR of HR regions

Primer	*Polarity*	*Sequence (5'- 3')*	*product*
2006	+	GCGGATCCGCATATAGGTTCAATGG (SEQ ID NO:__)	HR1
2007	-	CGAATTCATGTAATTAACCTGTCAA (SEQ ID NO:__)	HR1, HR1a, HR1b
2008	+	GCGGATCCAACCAAAAACAAATCGC (SEQ ID NO:__)	HR1a, HR1c
2009	+	GCGGATCCAACCAGAATGCTCAAGC (SEQ ID NO:__)	HR1b
2010	-	CGAATTCATTGTTTAACAAGTGTGT (SEQ ID NO:__)	HR1c
1998	+	CGAATTCACTCATATTTTCCCAATT (SEQ ID NO:__)	HR2
1999	+	GCGGATCCGAGCTTGACTCATTCAA (SEQ ID NO:__)	HR2-1, HR2-8, HR2-9, HR2-10
2064	+	GCGGATCCTTCAAAGAAGAGCTGGA (SEQ ID NO:__)	HR2-2
2065	+	GCGGATCCCTGGACAAGTACTTCAA (SEQ ID NO:__)	HR2-3
2066	+	GCGGATCCTTCAAAAATCATACATC (SEQ ID NO:__)	HR2-4
2067	+	GCGGATCCACATCACCAGATGTTGA (SEQ ID NO:__)	HR2-5
2068	+	GCGGATCCGTTGATCTTGGCGACAT (SEQ ID NO:__)	HR2-6
2069	+	GCGGATCCGACATTTCAGGCATTAA (SEQ ID NO:__)	HR2-7
1998	-	CGAATTCACTCATATTTTCCCAATT (SEQ ID NO:__)	HR2-1 – HR2-7
2034	-	CGAATTCATTTAATATATTGCTCAT (SEQ ID NO:__)	HR2-8
2070	-	CGAATTCACAATTCTTGAAGGTCAA (SEQ ID NO:__)	HR2-9
2071	-	CGAATTCAGTCAATGAGTGATTCAT (SEQ ID NO:__)	HR2-10
2072	+	GATCAGACTACAAGGATGACGATGACA AAG (SEQ ID NO:__)	FLAG-tag
2073	-	GATCCTTTGTCATCGTCATCCTTGTAGT CT (SEQ ID NO:__)	FLAG-tag